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14. ABSTRACT

Adenosine 2A receptor agonists when administered intrathecally produces long duration reversal of pain/allodynia in multiple animal models of neuropathic pain. To date we have tested central neuropathy(spinal nerve avulsion), spinal nerve ligation (L5/6 nerve ligation) and a model of sciatic inflammatory neuropathy (SIN). In all of these models the A2AR agonists produces long duration (>4 wk) of pain reversal from a single bolus injection. In addition, we have explored whether the time after injury that the drug is delivered affects the efficacy of the drug. To date, regardless of whether the drug is given in acute neuropathic pain (2 wk) or in well-established neuropathic pain (6 wk after injury), the drug is as effective in both extent of pain reversal and duration. We have begun to explore whether delivery of the drug at the site of injury (perisciatic) is able to reverse the allodynia and the preliminary data looks promising. Lastly, we have begun preliminary studies to identify whether blocking IL-10 at various times after A2AR agonist delivery reverses the efficacy of the drug on pain reversal.

15. SUBJECT TERMS- adenosine, interleukin 10, pain, anti-inflammatory, long duration

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Table of Contents

Page

Introductioné .é é é ..é ..3

Body…….3

Key Research Accomplishments……..14

Reportable Outcomes……14

Conclusion……14

References……15

Introduction

Neuropathic pain, resulting from nerve injury or inflammation, affects approximately 4 million people in the USA alone (*1*) and remains poorly managed by currently available therapeutics. Most of these therapeutics specifically target neurons. However, it is now known that spinal glia (astrocytes and microglia) play an important role in facilitating and maintaining neuropathic pain in animal models (*2*). We have identified a novel therapeutic target in adenosine 2_A receptors that modulate the immune cells within the CNS such that they switch from a classically pro-inflammatory state to an alternatively activated IL-10 generating state. The behavioral outcome of such a phenotypic switch results in a reversal of allodynia induced by neuropathic injury in rats for at least 4 wks from a SINGLE bolus administration. The purpose of this grant is to provide further evidence that this remarkable therapeutic effect can be translated to numerous animal models of neuropathic pain and to elucidate the underlying mechanisms that result in the production of IL-10 and subsequent reversal of the allodynia.

Body

Task 1. Obtain approval from the University of Colorado Institutional Animal Care & Use Committee (IACUC) for all animal work in the proposal

Task 1 has been completed and animal research has been conducted

Task 2. Aim IA1. Spinal Nerve Ligation (SNL): reversal of acute and chronic traumatic neuropathic pain by intrathecal (IT) ATL313.

Task 2 has been completed.

We have shown that a single intrathecal administration of ATL313 2 wk after spinal nerve ligation is able to reverse the neuropathic allodynia as evident by Figure 1 below. We have tested 2 doses of ATL313 and found that a higher dose (5 ng) than that required for chronic constriction injury (0.5 ng) reverses the allodynia for 4 wk. We also proposed to look at established neuropathic pain in the spinal nerve ligation model. Given the evidence of increased duration of action of ATL313 in the chronic constriction injury model relative to the spinal nerve ligation model, we have conducted the proposed experiment in the chronic constriction injury model as this provides a stronger test. The groups are now complete and the results show that ATL313 is as effective in established neuropathic pain as it is in acute neuropathic pain, as presented in Figure 2 below. These data show that the enduring effects of ATL313 on pain reversal are consistent across different pain models and that ATL313 is equally effective when administered shortly after the induction of pain as well as when administered during chronic neuropathic pain. The clinical relevance of this is incredibly important since many pain patients do not seek medical interventions until the pain has endured for weeks or even months.

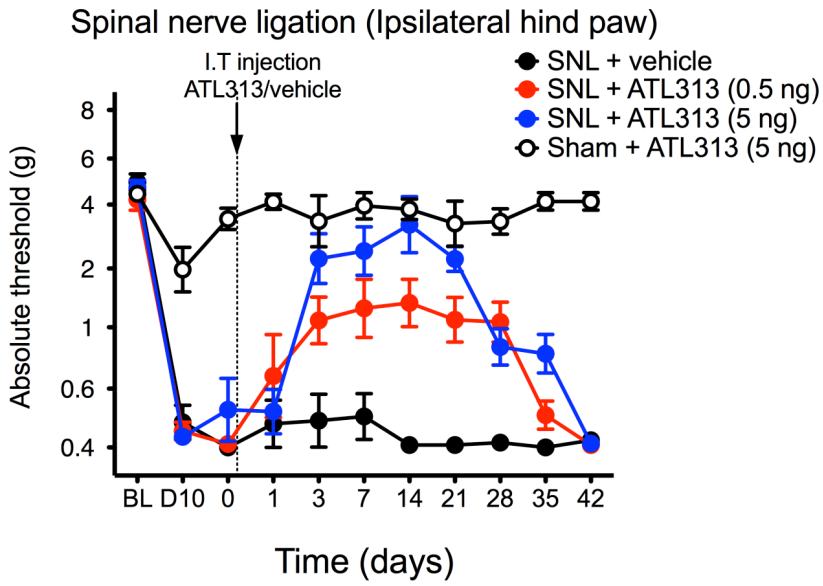

Figure 1. Spinal nerve ligation injury was induced at the L5 spinal level. Two weeks after surgery, a single intrathecal dose of ATL313 (0.5 ng or 5 ng) or vehicle was given. Mechanical allodynia was tested on the ipsilateral hind paw before surgery, before and after intrathecal drug delivery, and for 6 wks post-injection. ATL313 reversed the allodynia induced by chronic constriction injury from 3-28 days after drug administration ($P<0.05$, 2-way-repeated measures ANOVA).

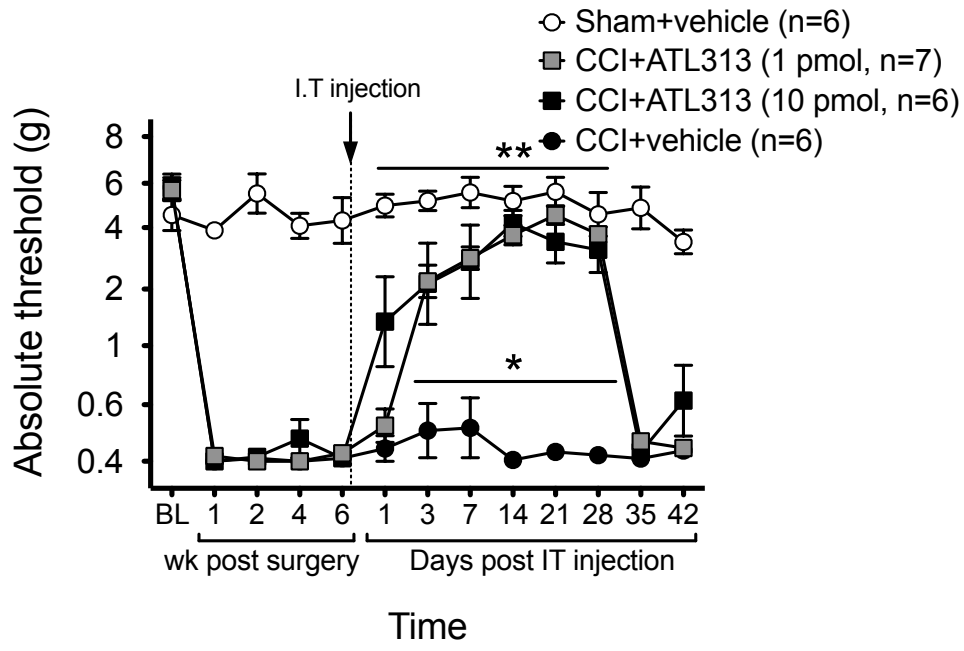

Figure 2. Chronic constriction injury was induced in the left sciatic nerve at the level of the mid thigh. 6 weeks after surgery, a single intrathecal dose of ATL313 (1 pmol or 10 pmol) or vehicle was given. Mechanical allodynia was tested on the ipsilateral hind paw before surgery, before and after intrathecal drug delivery and for 6 wks post-injection. ATL313 significantly reversed the allodynia induced by chronic constriction injury from 3-28 days after drug administration ($P<0.05$, 2-way-repeated measures ANOVA).

<u>Task 3</u>. Aim IB1. Sciatic Inflammatory Neuropathy (SIN): reversal of inflammatory neuropathic pain by IT vs. peri-sciatic nerve ATL313.

Task 3 has been completed.

Figures 3 and 4 below show reversal of SIN induced allodynia when ATL313 is delivered either peri-sciatically or intrathecally 24 h after the first dose of zymosan. We have established that chronic allodynia can be maintained by suspending the zymosan in saline as opposed to the conventional incomplete Freund's adjuvant, and all further studies will use saline. These data show the ability of ATL313 to reverse pain not only in a different peripheral pain model, but also its ability to reverse pain using different administration

routes (intrathecal and peri-sciatic). Again the clinical relevance of this important in that every pain patient is different and may not be able to or want to use certain routes of administration, and thus being able to use different routes and get the same efficacy is desirable.

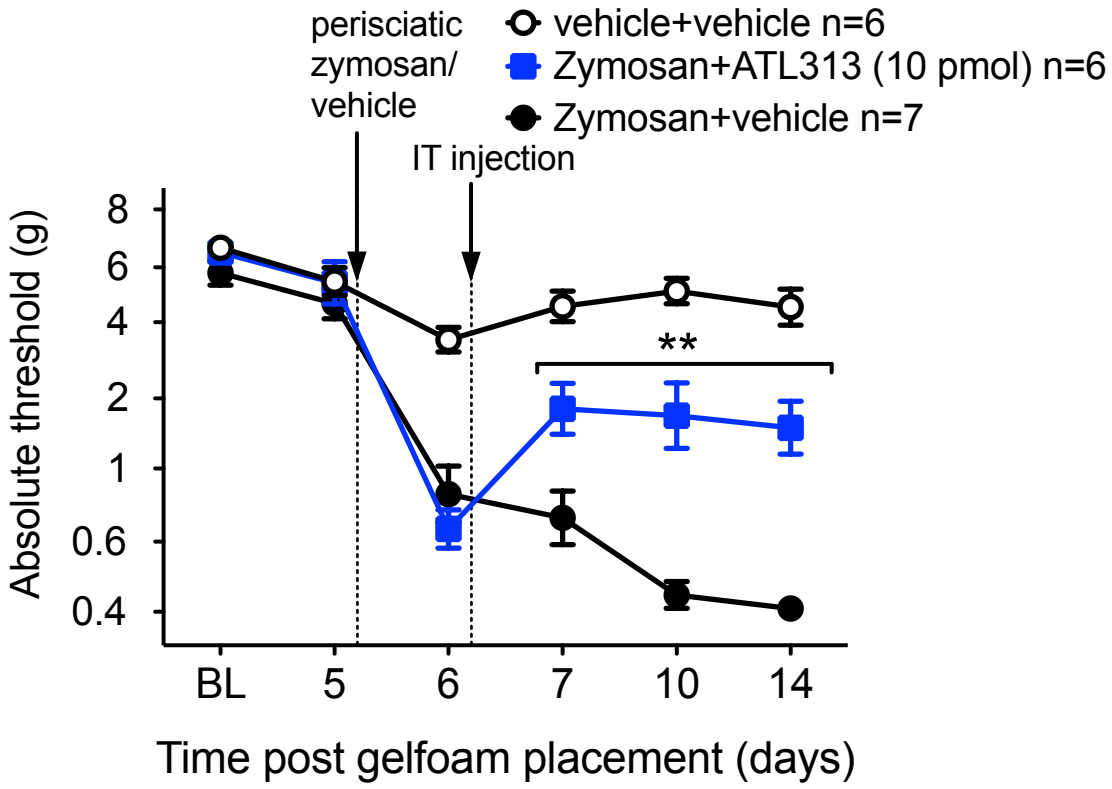

Figure 3. Gel foam was placed perisciatically to allow for zymosan delivery around the sciatic nerve at the mid thigh level. The rats were allowed to recover for 5 days from the gel foam placement surgery before zymosan was delivered. 5 days after surgery, 160 µg zymosan in 50 µl incomplete Freund's adjuvant was delivered through the catheter to the gel foam surrounding the sciatic nerve. Mechanical thresholds of the ipsilateral hind paw were tested before surgery, before zymosan delivery and 24 h after zymosan delivery. In rats displaying allodynia from the zymosan, rats were injected intrathecally with ATL313 or vehicle. Zymosan administration was continued every alternate day in order to maintain mechanical allodynia for 8 days. Intrathecal ATL313 significantly reversed the mechanical allodynia induced by zymosan for the duration of the experiment ($P<0.05$, 2-way repeated measures ANOVA).

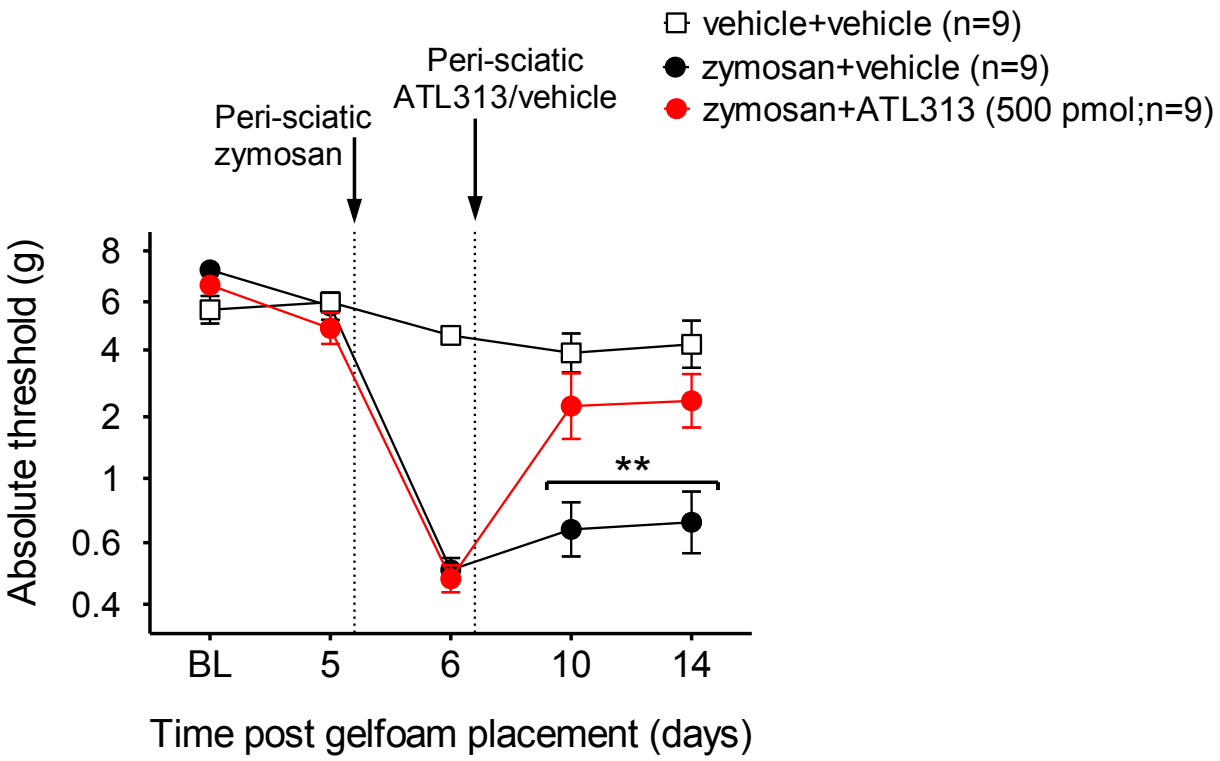

Figure 4. Baseline measures on the von Frey test for mechanical allodynia were performed before surgery. Sciatic inflammatory neuropathy (SIN) surgery was performed and rats were allowed to recover for five days. At five days post-surgery, following von Frey testing, zymosan (3.2 ug/ul) or saline vehicle was injected peri-sciatically. Twenty four hours later, again following von Frey testing, ATL313 (500 pmol) or DMSO vehicle was injected peri-sciatically. Peri-sciatic zymosan or saline vehicle was administered every other day for 7 days in order to maintain allodynia. Rats were tested on von Frey for mechanical allodynia at 10 and 14 days post-surgery. Peri-sciatic ATL313 significantly reversed zymosan-induced mechanical allodynia ($P<0.0001$, 2-way repeated measures ANOVA).

Task 4. Aim IB2. Spinal Cord Injury (SCI): reversal of acute and chronic central neuropathic pain by IT ATL313.

Task 4 has been completed.

We have shown that a single IT administration of an $A_{2A}R$ agonist (1 uM) 4 wk and 7 wk after T13/L1 spinal avulsion injury, what we have termed spinal neuropathic avulsion pain (SNAP), is able to completely reverse neuropathic allodynia as evident by Figures 5 and 6 below. What is remarkable about this is that SNAP spinal cord injury (SCI) is a *central* neuropathic pain model, whereas all of the previous tasks in this grant were on peripheral neuropathy models. Neuropathic pain can be from central or peripheral origin, or both, so it is important to develop treatments that are effective in both types of neuropathic pain. These data are also interesting in that $A_{2A}R$ agonism still reverses *established* central neuropathic pain (7 wks of robust, stable allodynia), which is again clinically important since neuropathic pain patients often do not seek treatment until after they have had the pain for weeks to months.

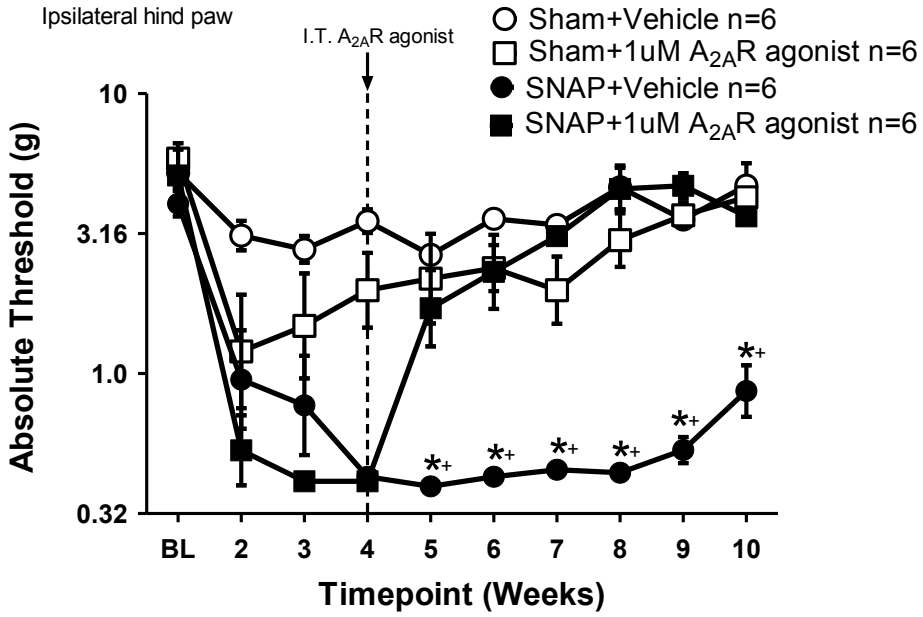

Figure 5. Unilateral T13/L1 avulsion induces mechanical allodynia as assessed by von Frey testing. An A_{2A}R agonist given as a single IT injection at 4 wk post-surgery reverses SCI-induced mechanical allodynia for at least 6 wk after administration ($p<0.05$). A_{2A}R agonist had no effect on sham-operated rats. Data are presented as mean ± SEM and analyzed using two-way repeated measures ANOVA. *$p<0.05$ SCI plus Vehicle compared to SCI plus A_{2A}R agonist; +$p<0.05$ SCI plus vehicle compared to Sham plus vehicle and Sham plus A_{2A}R agonist.

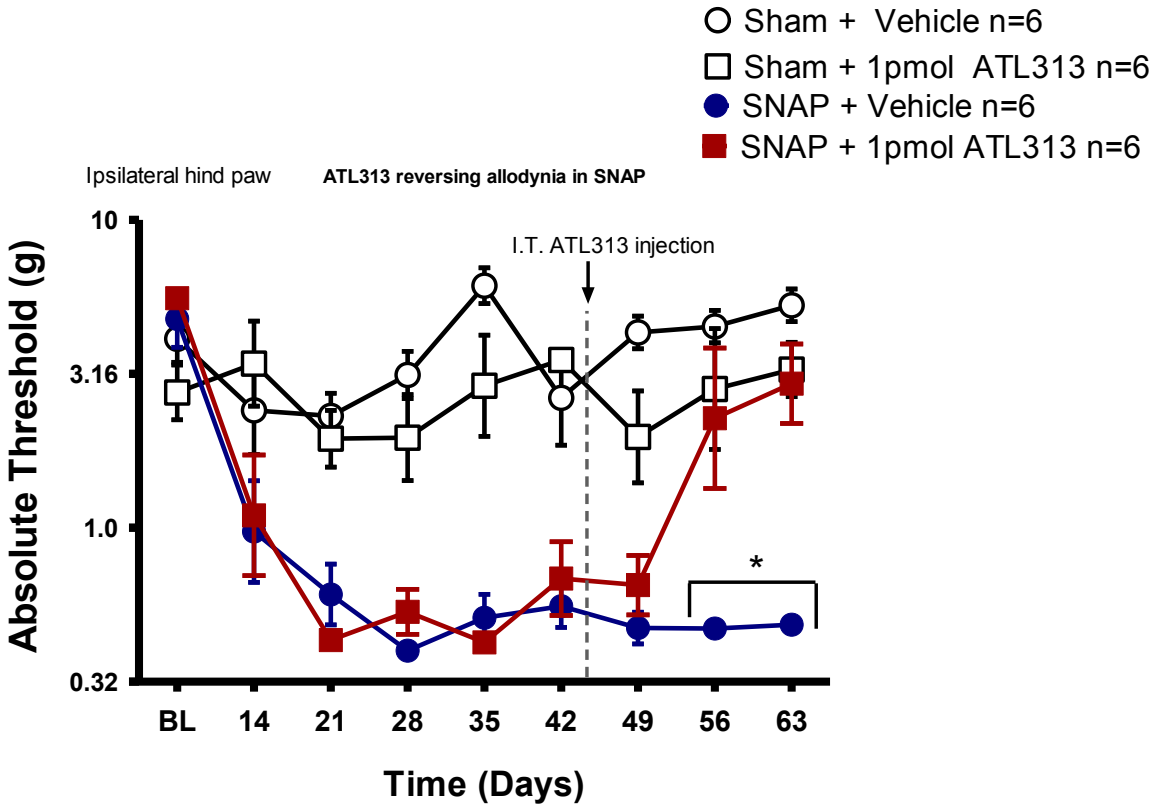

Figure 6. Unilateral T13/L1 avulsion SNAP or sham surgery with dura suture was performed and rats were allowed to recover for two weeks. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At seven weeks post-surgery, a single bolus injection of 1pmol ATL313 or DMSO vehicle was administered intrathecally. Beginning two weeks post-surgery (one week post-ATL313 administration), rats were tested for mechanical allodynia weekly for nine weeks, at which point allodynia from the SNAP surgery is resolved. Intrathecal ATL313 significantly reversed the mechanical allodynia induced by SNAP surgery ($P<0.0001$, 2-way-repeated measures ANOVA).

<u>Task 5.</u> AimIB3i. SIN: Prevention of inflammatory neuropathic pain by IT vs. peri-sciatic ATL313.

We have just begun this task; it is part of the last quarter of year 2. We know from Task 3 that ATL313 *reverses* zymosan-induced allodynia when it is administered both intrathecally and peri-sciatically. Here we are administering the zymosan and ATL313 on the same day to see if it will prevent the induction of allodynia. Figure 7 below shows that peri-sciatically administered ATL313 appears to be attenuating the initiation of zymosan-induced allodynia, although the group numbers are small, to date, and being added to in ongoing studies. We are continuing to fill in these groups and will also begin intrathecal administration of ATL313. This task and the others involving the SIN model have proven to be a bit difficult to complete. The research associate that began work on this grant, Lisa Loram, abruptly left the lab to accept a private sector position that suddenly became available and she had insufficient time prior to leaving to teach this model to the personnel taking over for her. It has taken time to get everyone trained up but we are back on track. The SIN model is somewhat difficult to work with in general because of how easy it is for the rats to get infections from the nature of the surgery, and so more rats may need to be used than with the other pain models (CCI, SNL, SNAP) in order to get complete groups. We are presently exploring a collaboration with the creator of this model, Dr. Erin Milligan (who created this model in Dr. Watkins' lab when Milligan was a graduate student here) to resolve the remaining optimization of the model and resolve the problems currently causing too large of a subject loss from sciatic catheter issues. The anticipate the remaining problems to be successfully resolved near term.

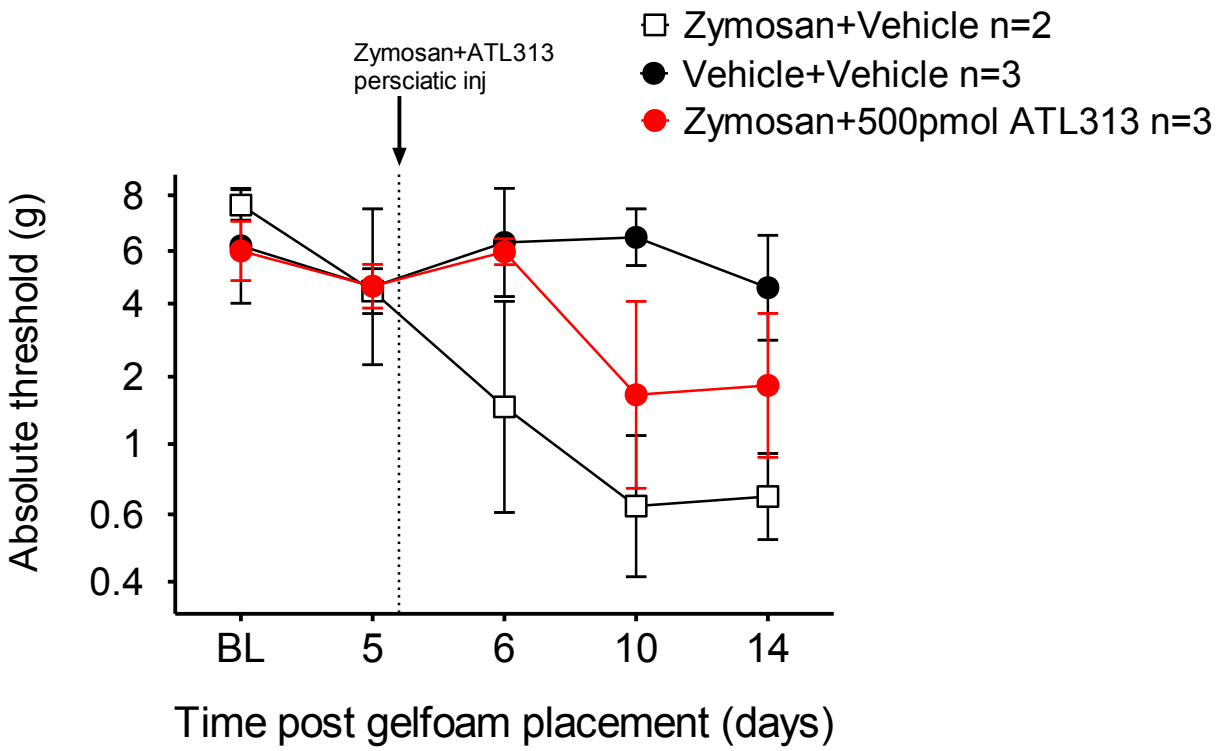

Figure 7. Baseline measures on the von Frey test for mechanical allodynia were performed before surgery. Sciatic inflammatory neuropathy (SIN) surgery was performed and rats were allowed to recover for five days. At five days post-surgery, following von Frey testing, zymosan (3.2 ug/ul) or saline vehicle and ATL313 (500pmol) or DMSO vehicle was injected peri-sciatically. Peri-sciatic zymosan or saline vehicle was administered every other day for 7 days in order to maintain allodynia. Rats were tested on von Frey for mechanical allodynia at 6, 10, and 14 days post-surgery. Although the study is not yet complete and there are, to date, small group numbers, it appears likely that ATL313 will likely be found to be able to delay and possibly partially attenuate zymosan-induced allodynia.

<u>Task 6.</u> Aim IB3ii. SCI: prevention of central neuropathic pain by ATL313.

Task 6 has been completed.

In Task 4 we showed that $A_{2A}R$ agonism is able to reverse both acute and chronic central neuropathic pain. Here we are able to show that $A_{2A}R$ agonism is also able to virtually abolish the induction of allodynia when administered 1 wk post-SCI SNAP surgery as seen in Figure 8 below. Although ATL313 was not able to completely prevent allodynia, it was still able to significantly attenuate the reduced pain thresholds compared to controls with the exception of a single timepoint (week 3). This is somewhat mirroring our initial findings in Task 5 where ATL313 is not completely preventing zymosan-induced allodynia but is attenuating the induction, here

dramatically so. It will be interesting to see whether that pattern stays consistent when we add more animals to the groups in Task 5. Even if ATL313 cannot completely prevent allodynia, its efficacy is far more than what many of the current pain treatments can do.

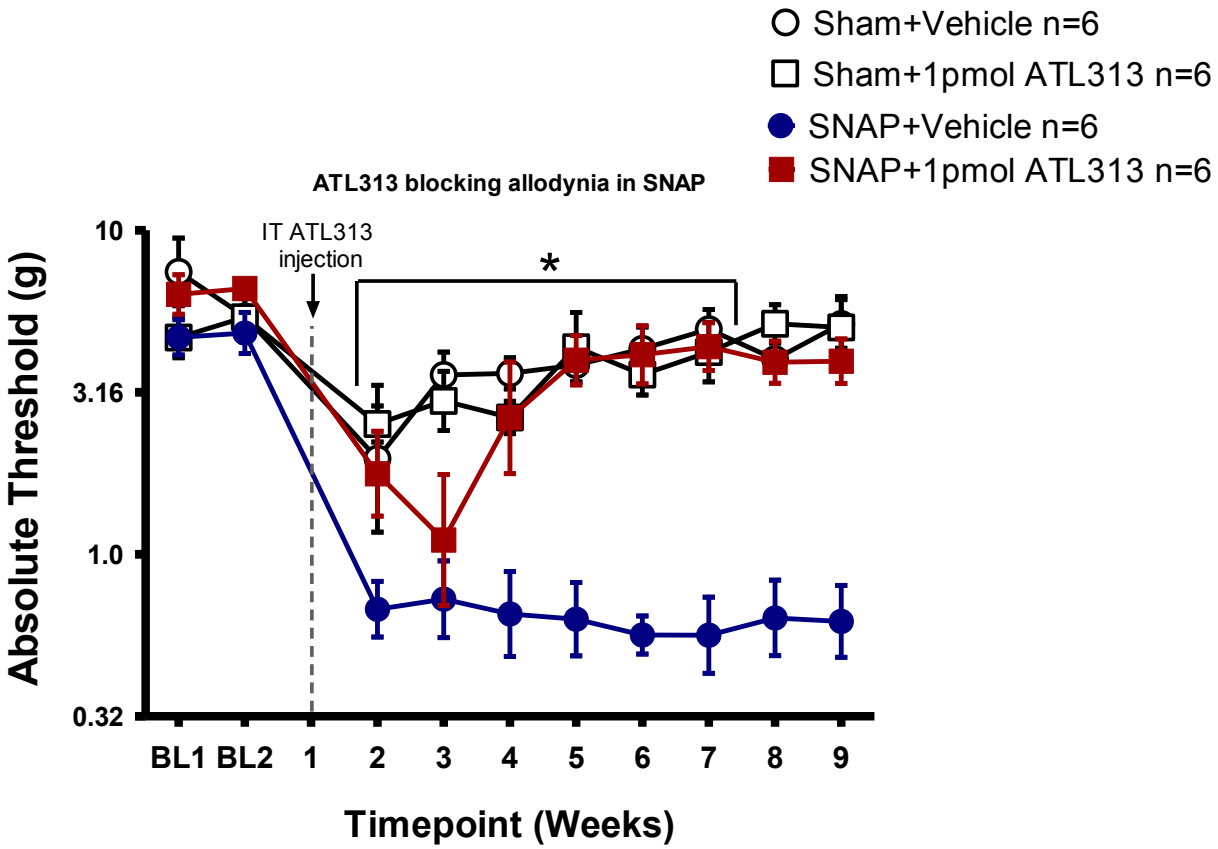

Figure 8. Unilateral T13/L1 avulsion SNAP or sham surgery with dura suture was performed and rats were allowed to recover for one week before ATL313 administration. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. At one week post-surgery, a single bolus injection of 1pmol ATL313 or DMSO vehicle was administered intrathecally. Rats were not tested for mechanical allodynia before ATL313 administration as reliable behavior is not obtained in SNAP rats until two weeks post-surgery after all spinal cord swelling has been resolved. Beginning two weeks post-surgery (one week post-ATL313 administration) rats were tested for mechanical allodynia weekly for seven weeks, at which point allodynia from the SNAP surgery is resolved. Intrathecal ATL313 significantly reversed the mechanical allodynia induced by SNAP surgery from week 2 through week 7 post-surgery (week 1 through week 7 post-ATL313) ($P<0.05$, 2-way repeated measures ANOVA).

<u>Task 7.</u> Aim IIA1. SNL: characterizing the involvement of interleukin(IL)-10 across the timecourse of effect.

Task 7 has been completed.

Given the evidence of increased duration of action of ATL313 in the SCI SNAP model relative to the spinal nerve ligation model, we have conducted the proposed experiment in the SCI SNAP model as this provides a stronger test. Figure 9 below shows that anti-IL-10 treatment 1 wk after $A_{2A}R$ agonist administration significantly abolishes the pain-relieving effects of the agonist. This indicates that the anti-inflammatory cytokine IL-10 is critically involved in the mechanism by which $A_{2A}R$ agonists exert their anti-allodynic effects, at least in the first 1-2 wks post-agonist administration. A second injection of anti-IL-10 two weeks after the first anti-IL-10 injection is problematic in its interpretation as there was a fall in pain thresholds for both groups. This may reflect an increased proinflammatory response to injected foreign protein (antibodies raised in a different animal species), although this is speculation at present. Regardless, the anti-IL10 did not create more allodynia then control IgG so, given what was observed was not a basement effect (i.e. more allodynia was possible to observe, had it occurred), the data below do not support that IL-10 is responsible for the ongoing allodynia later in the timecourse. If true, this raises the intesting question of what has taken over for IL-10 to maintain ongoing resolution of allodynia.

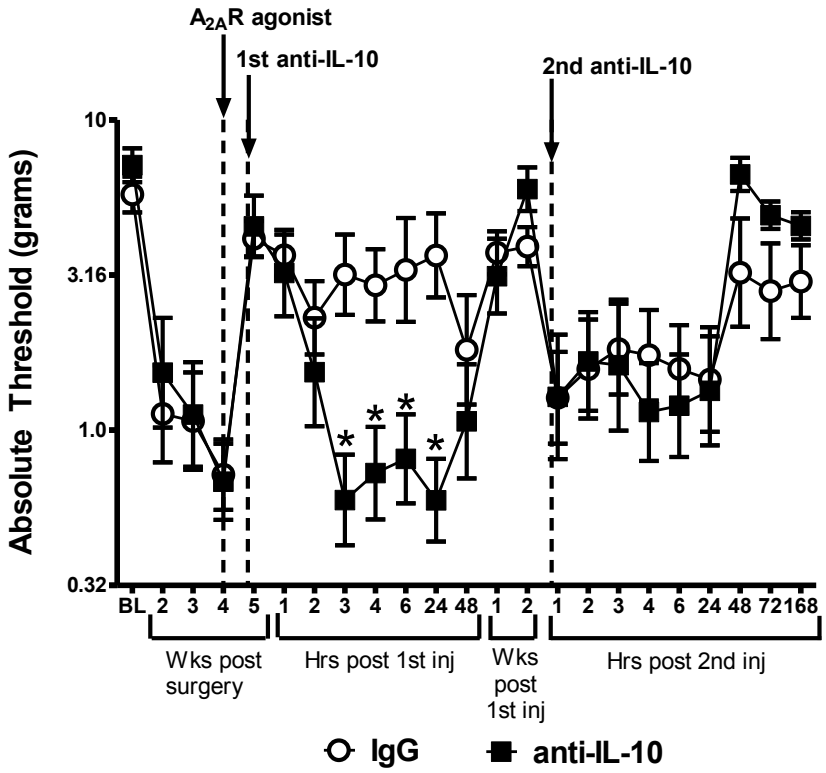

Figure 9. Unilateral T13/L1 avulsion SNAP surgery with dura suture was performed and rats were allowed to recover for two weeks. Mechanical thresholds of the ipsilateral hind paw were tested before surgery. Beginning two weeks post-surgery, rats were tested for mechanical allodynia weekly. At four weeks post-surgery, a single bolus injection of a 1uM $A_{2A}R$ agonist was administered intrathecally to all rats. One week later rats received a single injection of sheep anti-rat neutralizing IL-10 IgG antibodies (0.2 ug/ml;10 ul) or equivolume and equidose sheep IgG (0.2 ug/ml; 10 ul) and behavior was tested hourly for 6 hrs and then again at 24 and 48 hrs. Rats were then tested weekly for 3 wks and again injected with either IgG or anti-IL-10 and tested hourly for 6 hrs and then again at 24, 48, 72, and 168 hrs. Anti-IL-10 significantly abolished the effects of the $A_{2A}R$ agonist beginning 4 hrs after the injection and this effect lasted for 48 hrs (interaction, $F_{(6,72)}=4.808$, $p<0.001$; n=9 per group). Behavior returned to pre-anti-IL-10 or IgG levels 2 wk later. IgG had no effect on behavior. A second injection of either anti-IL-10 or IgG administered 3 wk later did not have a significant effect on allodynia behavior (interaction, $F_{(7,84)}=1.007$, $p>0.05$; n=9 per group) . Data are presented as mean ± SEM and analyzed using two-way repeated measures ANOVA. *$p<0.05$ SCI plus IgG compared to SCI plus anti-IL-10.

<u>Task 9.</u> Aim IIB1. SNL: protein kinase (PK) C/PKA involvement with acute and chronic peripheral neuropathic pain.

We have just begun working on this task in the last quarter of year 2. Given the evidence of increased duration of action of ATL313 in the chronic constriction injury model relative to the spinal nerve ligation model, we have conducted the proposed experiment in the chronic constriction injury model as this provides a stronger test. Here we administered protein kinase A (PKA) and protein kinase C (PKC) inhibitors to determine the involvement of PKA and PKC in the mechanism by which $A_{2A}R$ agonists exert their anti-allodynic effects. Figure 10 below shows that the PKC inhibitor chelerythrine abolishes the anti-allodynic effects of the $A_{2A}R$ agonist, similar to that of anti-IL-10. Figure 11 below shows a similar pattern with the PKA inhibitor H-89. There is some controversy in the literature as to whether or not PKA and PKC inhibitors are proinflammatory or anti-inflammatory when administered *in vivo*. We are currently trying to review the literature as thoroughly as possible in order to determine whether or not we will be able to reliably and confidently interpret the data we collect from these experiments as the literature is also now complex (although rarely if ever discussed in in vivo studies) as to interpretation of all such compounds when administered in vivo as there are suspected off target effects on various cell types present in vivo that complicate knowing the meaningfulness of results obtained. We are presently carefully searching the literature on these types of compounds (which are frequently used in vivo without discussion of potential interpretational issues) to define how best to proceed.

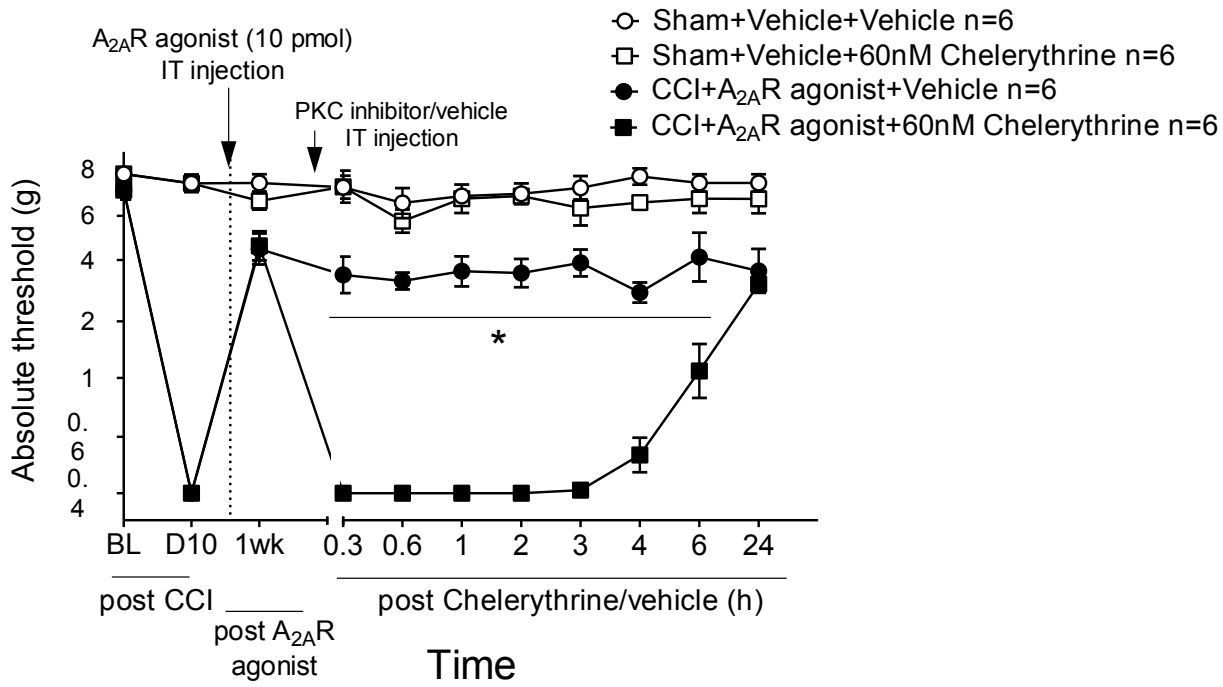

Figure 10. Chronic constriction injury was induced in the left sciatic nerve at the level of the mid thigh. Two weeks after surgery, a single intrathecal dose of an $A_{2A}R$ agonist or vehicle was given. Mechanical allodynia was tested on the ipsilateral hind paw before surgery, before and after intrathecal drug delivery. One week later, a single intrathecal injection of the PKC inhibitor chelerythrine (60nM) was administered and behavior was assessed 0.3, 0.6, 1, 2, 3, 4, 6 and 24 hrs post injection. Chelerythrine signifcantly abolished the anti-allodynic effect of the $A_{2A}R$ agonist beginning 0.3 hrs post-injection and for up to 6 hrs post-injection ($P<0.05$, 2-way-repeated measures ANOVA).

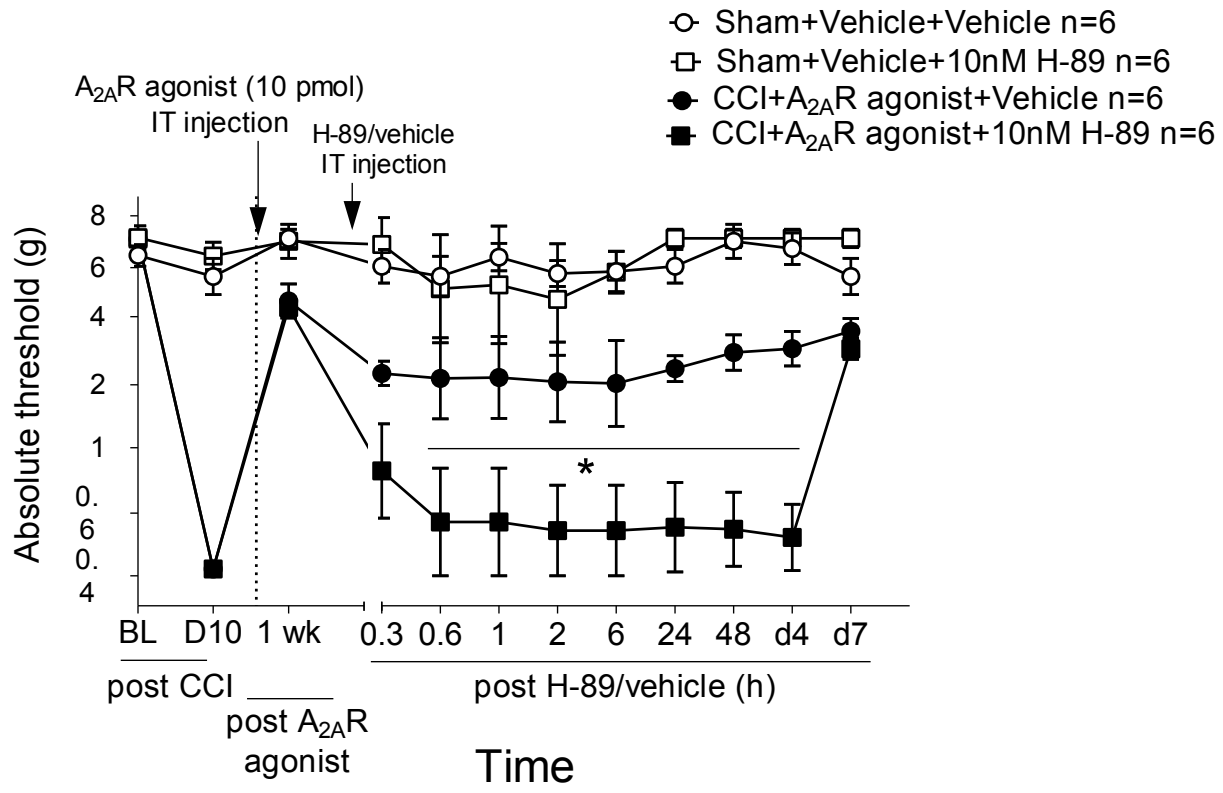

Figure 11. Chronic constriction injury was induced in the left sciatic nerve at the level of the mid thigh. Two weeks after surgery, a single intrathecal dose of an $A_{2A}R$ agonist or vehicle was given. Mechanical allodynia was tested on the ipsilateral hind paw before surgery, before and after intrathecal drug delivery. One week later, a single intrathecal injection of the PKA inhibitor H-89 (10nM) was administered and behavior was assessed 0.3, 0.6, 1, 2, 3, 4, 6, 24, 48 hrs, 4, and 7 days post-injection. H-89 significantly abolished

the anti-allodynic effect of the $A_{2A}R$ agonist beginning 0.6hrs post-injection and for up to 4 days post-injection ($P<0.05$, 2-way-repeated measures ANOVA).

Key Research Accomplishments

- One manuscript has been submitted for publication and is under review
- Ellis has begun the initial stages of the manuscript for publication and will continue throughout the next few quarters.
- The work was presented at the annual Society for Neuroscience conference in Washington DC in November 2011 and in New Orleans, LA in November 2012.

Reportable outcomes

- The effect of $A_{2A}R$ agonism on spinal cord injury has been successfully presented as Amanda Ellis' Master's degree which she received in January 2012.
- One manuscript has been submitted for publication and was rejected but is currently under review in a different journal
- Ellis has begun the initial stages of the manuscript for publication and will continue throughout the next few quarters.
- Abstract was accepted (*3*) and the work was presented at the annual Society for Neuroscience conference in New Orleans, LA in November 2012.

Conclusions

We continue to make good progress and have maintained the required outputs and data collection according to the statement of work. ATL313 continues to present as a novel compound producing remarkably long duration of reversal of pain from a single administration. $A_{2A}R$ agonism both prevents and reverses acute and chronic neuropathic pain, and does so in neuropathies of both central (SCI SNAP) and peripheral (CCI, SNL, SIN) origin. This is important clinically since many neuropathic pain patients, with both central and peripheral neuropathies, do not seek treatment for weeks or month after the onset of pain. Furthermore, the anti-allodynic effects seen with $A_{2A}R$ agonism are consistent with different routes of administration (peri-sciatic, intrathecal). Pain patients are not always comfortable with certain routes of administration, and thus having equal efficacy using different routes is desirable. We have also shown that the anti-inflammatory cytokine IL-10 is initially involved in the mechanism by which $A_{2A}R$ agonists exert their pain relieving effects, and likely PKA and PKC, although more work needs to be done in order to be able to interpret those results. Taken together, all of these data suggest that ATL313 would be a successful new neuropathic pain treatment. At the same time, it is important to continue investigating the underlying mechanisms of this remarkable drug compound in order to use it most effectively. We continue to thank the Department of Defense for their continued support of the project and hope they find the outcome of the project to date exciting and novel with potential clinical relevance down the road.

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2. **L. R. Watkins *et al.*, *Brain, behavior, and immunity* 21, 131 (Feb, 2007).**
3. **A. L. Ellis, in *Society for Neuroscience*. (New Orleans, LA, 2012).**